

Identification and biochemical characterization of Asp t 36, a new fungal allergen from *Aspergillus terreus*

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Aspergillus terreus is an allergenic fungus, in addition to causing infections in both humans and plants. However, the allergens in this fungus are still unknown, limiting the development of diagnostic and therapeutic strategies. We used a proteomic approach to search for allergens, identifying 16 allergens based on two-dimensional immunoblotting with *A. terreus* susceptible patient sera. We further characterized triose-phosphate isomerase (Asp t 36), one of the dominant IgE (IgE)-reactive proteins. The gene was cloned and expressed in *Escherichia coli*. Phylogenetic analysis showed Asp t 36 to be highly conserved with close similarity to the triose-phosphate isomerase protein sequence from *Dermatophagoides farinae*, an allergenic dust mite. We identified four immunodominant epitopes using synthetic peptides, and mapped them on a homology-based model of the tertiary structure of Asp t 36. Among these, two were found to create a continuous surface patch on the 3D structure, rendering it an IgE-binding hotspot. Biophysical analysis indicated that Asp t 36 shows similar secondary structure content and temperature sensitivity with other reported triose-phosphate isomerase allergens. *In vivo* studies using a murine model displayed that the recombinant Asp t 36 was able to stimulate airway inflammation, as demonstrated by an influx of eosinophils, goblet cell hyperplasia, elevated serum IgEs, and induction of Th2 cytokines. Collectively, our results reveal the immunogenic property of Asp t 36, a major allergen from *A. terreus*, and define a new fungal allergen more broadly. This allergen could serve as a potent candidate for investigating component resolved diagnosis and immunotherapy.

There has been a rapid increase in the global prevalence of allergies due to exposure to bio-pollutants generated from urbanization and industrialization (1). Among these bio-pollutants, fungal spores are the most abundant and can induce a severe allergic reaction in susceptible individuals upon inhalation (2). An atopic response occurs when allergens bind and cross-link IgE antibodies on the surfaces of mast cells and basophils via the FcεRI receptor. This leads to degranulation of the mast cells and the release of histamine, which activates a signaling cascade to generate Th2 cytokines (1). Sensitized individuals suffer from hypersensitivity against such fungal antigens causing rhinitis, hay fever, and allergic eczema.

In India, more than 25% of the total population exhibit some sort of atopic diseases due to aero-allergen exposure (3).

Among the aerospora, *Aspergillus* species are widely distributed in nature. Although the genus *Aspergillus* contains over 150 species, only a few are documented to be allergenic to humans. 39.5% of allergic patients are attributable to *Aspergillus* sensitization in India (4). According to aerobiological surveys, *Aspergillus terreus* is one of the most prevalent aerospora among the *Aspergillus* species and was found to be widespread in the agricultural area as well as in climate-controlled indoor environments (5). Besides its clinical evidence in causing allergic bronchopulmonary aspergillosis, there has been very limited study on its allergenicity (4).

Although allergic diseases are manageable partially through antihistamines or avoidance, there is no cure yet. Immunotherapies with purified allergens have shown some promise but are often complicated by adverse side effects. Mapping IgE-binding epitopes on the tertiary structure help in the rational design of allergens with reduced allergenicity. To aid this, the identification of allergens is a prerequisite from any allergenic source. In recent years, proteomic techniques have surpassed other methods as a useful tool for the identification of many fungal allergens because of its efficiency and speed (6). Proteins such as fibrinogen-binding protein (Asp f 2), peroxisomal membrane proteins (Asp f 3) from *Aspergillus fumigatus*; alkaline serine proteinase (Asp o 13) from *Aspergillus oryzae*; alkaline serine proteinase (Asp fl 13) from *Aspergillus flavus* were identified as a major allergen using proteomic platforms (7–10). Once identified, preparing recombinant allergens help in determining the structure and identifying possible epitopes (11). Thereafter, further studies can be done by manipulating the epitopes to develop hypoallergenic vaccines for immunotherapy regimes (12). As human experimentation is ethically restrictive, mice have served as models for many allergic diseases. Intranasal instillation with common indoor filamentous fungi was shown to induce allergic symptoms in the murine lungs (13). This not only helps to validate the atopicity of a novel allergen in an *in vivo* condition but also assists in understanding the molecular mechanism of hypersensitivity.

In this present study, we have identified the allergens from *A. terreus*, using a proteomics approach. Subsequently, the major allergen triose-phosphate isomerase (Asp t 36) was identified and purified both in its native state as well as in the recombinant form. Triose-phosphate isomerase, being widely reported as an allergen across crustaceans and shellfish (14, 15), makes it an interesting protein to study its epitopes, probable cross-reactivity, and evolution across species. The molecular

This article contains supporting information.

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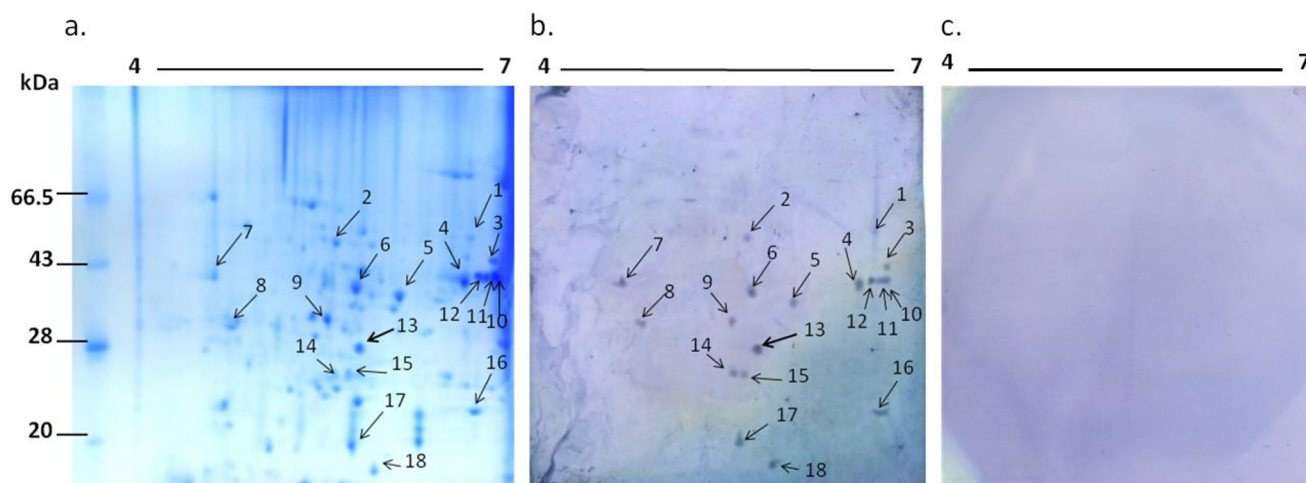


Figure 1. Serological determination of IgE reactive spots from the total proteome of *A. terreus*. a, 2D-PAGE of *A. terreus* protein profile in the 4–7 pI range. The allergenic protein spots are numbered after the confirmation through a 2D blot. b, 2D immunoblot of *A. terreus* protein with patients' serum pool ($n = 15$) in the 4–7 pI range (one representative blot) along with marked IgE reactive protein spots. c, 2D blot with healthy patients' serum pool ($n = 3$) in the 4–7 pI range (Control).

characterization of this protein as an allergen in fungus remains unsolved to date. An effort was made to identify linear epitopes as a step toward understanding its molecular basis of allergenicity. The recombinant version of Asp t 36 was assessed for its atopicity in a mouse model as well as *in vitro* experiments opening up possibilities to study the protein in more detail.

Results

Allergenecity assessment of *A. terreus* in the study subjects

The skin prick test was performed to assess the biopotency of fungal allergen among both asthmatic and nonasthmatic patients. 60% of asthmatic patients ($n = 86$) and 49% of nonasthmatic patients ($n = 174$) were found to show sensitivity against different tested fungal species. Among these tested species, *A. terreus* was found to be one of the important fungal allergens that showed their atopicity in both asthmatic and nonasthmatic patients. Patients' demographic details are shown in Table S1.

The prevalence of specific IgE levels and its reactivity with crude *A. terreus* protein was determined through ELISA and dot blot by using individual *A. terreus* hypersensitive patients' sera. All 15 sensitized patients' sera showed a high specific IgE level compared with controls. In ELISA, the optical density (O.D.) for the specific IgE absorbance values for atopic patients' sera was 4–10 times higher than that of the control sera (Table S1). In dot blot, the higher level of reactivity was found in atopic patients' sera with 2+ and 3+ skin prick test reactivity (Fig. S1a).

Detection of IgE reactive spots from the proteome of *A. terreus*

Protein extracted from a 14-day mycelial mat of *A. terreus* was run on 12% acrylamide gel. Twenty-four prominent bands were resolved in the molecular mass range of 14 to 66 kDa after Coomassie Brilliant Blue staining (Fig. S1b). SDS-PAGE immunoblots performed with the 15 atopic patients' sera revealed

more than 10 IgE reactive protein bands ranging between 14 and 50 kDa. The 28-kDa band was observed for most of the sensitive patients (Fig. S1b).

For a better resolution, the proteins from *A. terreus* were separated in a 2D gel. The total proteome of *A. terreus* showed a well-resolved protein profile in 12% 4–7pI 2D gel (Fig. 1a). On clear separation of individual protein spots, this gel was subsequently electrotransferred into a polyvinylidene difluoride membrane, followed by the immunostaining with individual and pooled sera. A total of 18 distinct IgE reactive protein spots (Fig. 1b) were detected using pooled sera. Spots 6, 13, and 17 showed a relatively higher intensity of IgE binding compared with other spots indicating them to be major allergens. Upon immunoblotting with individual sera, IgE reactivity frequency profile to individual spots showed spot 13 of 28 kDa and 5.6 pI to be the most sensitive and hence determined as a major allergen of *A. terreus* (Fig. S1c). No allergenic spots were detected in control immunoblot (Fig. 1c).

Identification of IgE reactive proteins by MS

Protein spots ($n = 18$) corresponding to allergens (Fig. 1b) were excised from the gel and analyzed by MALDI-TOF. Sixteen proteins could be confidently identified and are recorded in Table 1. These include glutamine synthetase (spot 2), phosphoglycerate kinase (spot 3), conserved hypothetical protein (spot 4), fructose-bisphosphate aldolase (spot 6), glucan 1,3- β -glucosidase precursor (spot 7), vacuolar protease A precursor (spot 8), proteasome component Y7 (spot 9), triose-phosphate isomerase (TPI; spot 13), heat shock protein 82 (spot 14), hypothetical protein ATEG_08454 (spot 15), S-(hydroxymethyl)GSH dehydrogenase (spot 16), hypothetical protein ATEG_02590 (spot 17), and predicted protein (spot 18). Three consecutive spots (spots 10, 11, 12) toward the basic pI range were identified as formate dehydrogenase. As *A. terreus* is a sequenced organism, most of the proteins were identified based on MS, whereas for a few MS/MS were also done to increase the confidence of identifications. Some of the proteins exhibited a difference in

Table 1

Identification of allergenic proteins through MALDI-TOF/TOF from *A. terreus*

Peptides are identified by MASCOT search engine tool. Because of low spectral quality, spots 1 and 5 could not be identified. The unmarked identified proteins include completely novel allergens (detailed analysis results are given in Table S2).

Sr. No.	Spot No.	Identified protein	Organism	NCBI accession No.	No. of unique peptides	Theoretical mass (kDa)/pI	Experimental mass (kDa)/pI	Score ($p < 0.05$)	Sequence coverage
1.	2.	Glutamine synthetase ^a	<i>A. terreus</i> NIH2624	EAU36469.1	2 (MS/MS)	38/5.49	50/5.48	100	4%
								61	6%
2.	3.	Phosphoglycerate kinase ^a	<i>A. terreus</i> NIH2624	EAU38870.1	3 (MS/MS)	44/6.58	43/6.78	76	3%
								82	4%
								110	4%
3.	4.	Conserved hypothetical protein	<i>A. terreus</i> NIH2624	EAU30461.1	2 (MS/MS)	37/5.84	39/6.22	90	4%
								63	8%
4.	6.	Fructose-bisphosphate aldolase ^a	<i>A. terreus</i> NIH2624	EAU35150.1	12 (MS)	39/5.58	38/5.63	81	43%
					3 (MS/MS)			57	3%
								133	6%
								161	6%
5.	7.	Glucan 1,3- β -glucosidase precursor	<i>A. terreus</i> NIH2624	EAU35651.1	6 (MS)	46/4.42	39/5.00	49	22%
6.	8.	Vacuolar protease A precursor	<i>A. terreus</i> NIH2624	EAU35123.1	3 (MS/MS)	43/4.78	32/5.02	123	4%
								213	4%
								92	5%
7.	9.	Proteasome component Y7	<i>A. terreus</i> NIH2624	EAU32891.1	1 (MS/MS)	30/5.55	31/5.45	70	7%
8.	10.	Formate dehydrogenase	<i>A. terreus</i> NIH2624	EAU33446.1	12 (MS)	46/8.14	41/6.9	93	41%
					3 (MS/MS)			87	3%
								128	5%
								78	5%
9.	11.	Formate dehydrogenase	<i>A. terreus</i> NIH2624	EAU33446.1	14 (MS)	46/8.14	41/6.8	89	43%
					3 (MS/MS)			54	3%
								96	5%
								83	5%
10.	12.	Formate dehydrogenase	<i>A. terreus</i> NIH2624	EAU33446.1	13 (MS)	46/8.14	41/6.7	66	42%
					1 (MS/MS)			75	5%
11.	13.	Triose-phosphate isomerase ^{a,b}	<i>A. terreus</i> NIH2624	EAU33924.1	8 (MS)	27/5.42	28/5.6	75	50%
					3 (MS/MS)			84	4%
								111	8%
								118	12%
12.	14.	Heat shock protein 82	<i>A. terreus</i> NIH2624	EAU31169.1	2 (MS/MS)	79/4.97	26/5.5	103	1%
								55	1%
13.	15.	Hypothetical protein ATEG_08454 ^a	<i>A. terreus</i> NIH2624	EAU31627.1	13 (MS)	26/5.61	26/5.62	78	62%
14.	16.	S-(hydroxymethyl)GSH dehydrogenase	<i>A. terreus</i> NIH2624	EAU34016.1	9 (MS)	33/8.64	24/6.6	54	19%
15.	17.	Hypothetical protein ATEG_02590	<i>A. terreus</i> NIH2624	EAU37552.1	2 (MS/MS)	38/5.78	19/5.62	64	3%
								47	6%
16.	18.	Predicted protein	<i>A. terreus</i> NIH2624	EAU37951.1	5 (MS)	11/5.61	11/5.7	39	21%

^a Identified as an allergen from fungal/other species.

^b Indicates the major allergen of *A. terreus*; reported as an allergen in fungus for the first time; validated by WHO/IUIS.

observed and theoretical molecular weight values. Two protein spots (1 and 5) could not be identified because of their low spectral quality.

Native purification of major allergen, TPI

A two-step chromatographic method incorporating sequential ion exchange followed by size exclusion was able to purify the protein in its native state. Total protein from the *A. terreus* fungal mat was extracted in a buffer with pH higher than the pI of TPI and loaded onto a strong anion exchanger HiTrap Q HP. An elution obtained at 0.8 M NaCl was collected and run on SDS-PAGE gel (Fig. 2, a and b). The fractions showing a high-intensity protein band at 28 kDa were pooled and concentrated by the sequential use of 10- and 30-kDa centrifugal filters. Finally, the concentrated protein was loaded on a Superdex 200 increase 10/300 GL gel-filtration column. Each of these steps resulted in lowering the concentration of native TPI (nTPI). From 4 g of the mycelial mat, 200 μ g of nTPI was obtained. The desired protein fractions, eluted from size exclusion column chromatography ran as a single band on SDS-PAGE, followed by loading on the 2D gel

of 3-10 pI (NL) range for checking its molecular weight as well as pI, respectively (Fig. 2, c-e). Its identification was re-checked by MS (Fig. S2). The allergenicity of the purified native protein was re-confirmed by ELISA, IgE immunoblotting is described later.

Cloning and overexpression of the allergen TPI

cDNA from *A. terreus*, was used as a template for PCR amplification using primers specific to TPI. The cDNA was cloned into the pET28a vector and expressed in *Escherichia coli* (Fig. 3a). Upon induction, recombinant TPI (rTPI) was found in the soluble fraction of the cell homogenate. After nickel affinity purification, the purified protein of interest was found near the desired molecular mass of 28 kDa without any visible contaminants. To further assess its quaternary structure, the purified protein was passed through a gel filtration column (Superdex 200). In a gel filtration chromatogram, a single peak at the monomer size was observed. A truncated product at 25 kDa was observed along with the matured protein in the SDS-PAGE (Fig. 3, b and c). However, upon analyzing by MS, both of them

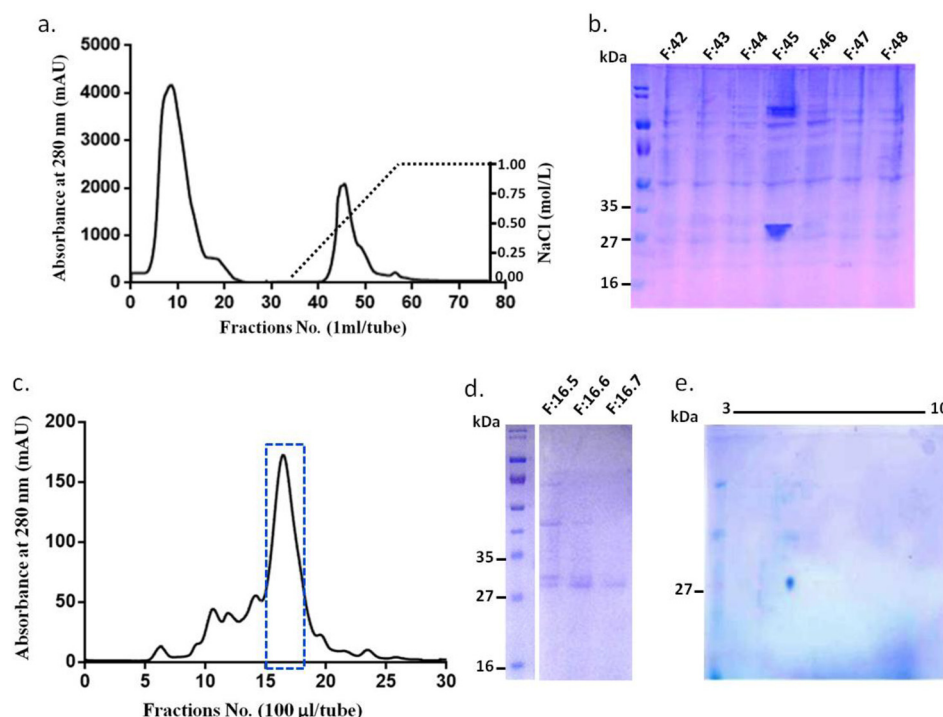


Figure 2. Chromatographic purification of nTPI protein from *A. terreus*. *a*, chromatogram of anion exchange chromatography (Q HP) using 20 mM Tris-HCl, pH 7, binding buffer and 1 M NaCl gradient. *b*, 12% SDS-PAGE image of the collected fractions containing desired 28-kDa nTPI protein band in F:45 after passing through Q HP. *c*, chromatogram of gel filtration chromatography with the pooled Q HP fractions containing protein of interest, getting from sequential passing through 10- and 30-kDa Amicon centrifugal filters. *d*, 12% SDS-PAGE image of the purified nTPI band in F:16.7 with the desired purity after gel filtration chromatography. *e*, 12% 2D gel image of the purified nTPI in 3–10 pI (NL) range for confirmation its molecular weight and pI.

showed significant identity to the desired protein sequence of TPI.

Immunological characterization of native and recombinant TPI

The binding efficiency of IgE antibodies to nTPI and rTPI was checked with 15 *A. terreus* allergic patients' sera by ELISA. Compared with control, both nTPI and rTPI could evoke a significant amount of IgE binding (both $p < 0.05$, Fig. 3*d*). Moreover, upon further investigation on the allergenicity of nTPI and rTPI through immunoblotting with *A. terreus*-sensitive pooled patient sera and rabbit anti-TPI polyclonal antibodies, both purified proteins were found to be immunoreactive (Fig. 3*e*). Immunoblotting with an isopropyl 1-thio- β -D-galactopyranoside-induced *E. coli* extract as a control showed IgE binding specificity only to the overexpressed protein (Fig. 3*e*). IgE binding was noticed for the two parts of the *E. coli*-expressed rTPI in IgE and IgG Western blotting, respectively. The full-length TPI protein was recognized as Asp t 36 by the WHO/IUIS allergen nomenclature committee.

Thermal stability of Asp t 36

The properly folded structure of nAsp t 36 as well as rAsp t 36 was observed at 25 °C by CD spectroscopy (Fig. 3*f*). The CD spectrum between nAsp t 36 and rAsp t 36 was found to be comparable indicating both of them to adopt a similarly folded secondary structure. Upon analysis in the Dichroweb server, the recombinant protein showed 44% of α -helix, 12% β -sheet, and 44% random coils. Upon monitoring temperature induced

unfolding, the β -sheet region of Asp t 36 started to denature from ~58 °C and became completely unfolded at 75 °C. On the contrary to β -helices, α -helices showed its stability up to 39 °C, whereas denaturation took place from ~48 °C and completely unfolded at 74 °C, respectively (Fig. 3*g*). On attempting to refold by cooling down from 90 to 25 °C, rAsp t 36 was not able to regain its original folded structure thereby suggesting the process to be irreversible.

Structural modeling and epitope analysis

Based on sequence similarity (sequence similarity, 0.47; sequence identity, 59.76%), the crystallographic structure of TPI from *Tenebrio molitor* (PDB code 2I9E) was used as a template for obtaining a homology-based model for Asp t 36 in the Swiss Model workspace (Fig. 4*a*). On alignment, Asp t 36 with the chosen template, the backbone root mean square deviation value was 0.123 Å. The main chain conformation was found to be in the acceptable region of the Ramachandran plot. PROCHECK analysis revealed that 96.3% of the amino acids are in the favored region and 3.7% in the allowed region for this predicted model. The homology model structure of Asp t 36 revealed 12 α -helices and 8 β -strands with alternating turns. The secondary structure of Asp t 36 is illustrated in Fig. 4*b*.

Six linear epitopes (E1, aa 58–78; E2, aa 88–113; E3, aa 131–150; E4, aa 170–188; E5, aa 190–209; and E6, aa 222–239) were selected based on their prediction of high antigenicity from four online prediction tools. They were mapped on the surface filled model of Asp t 36. Indirect ELISA and dot blot were done

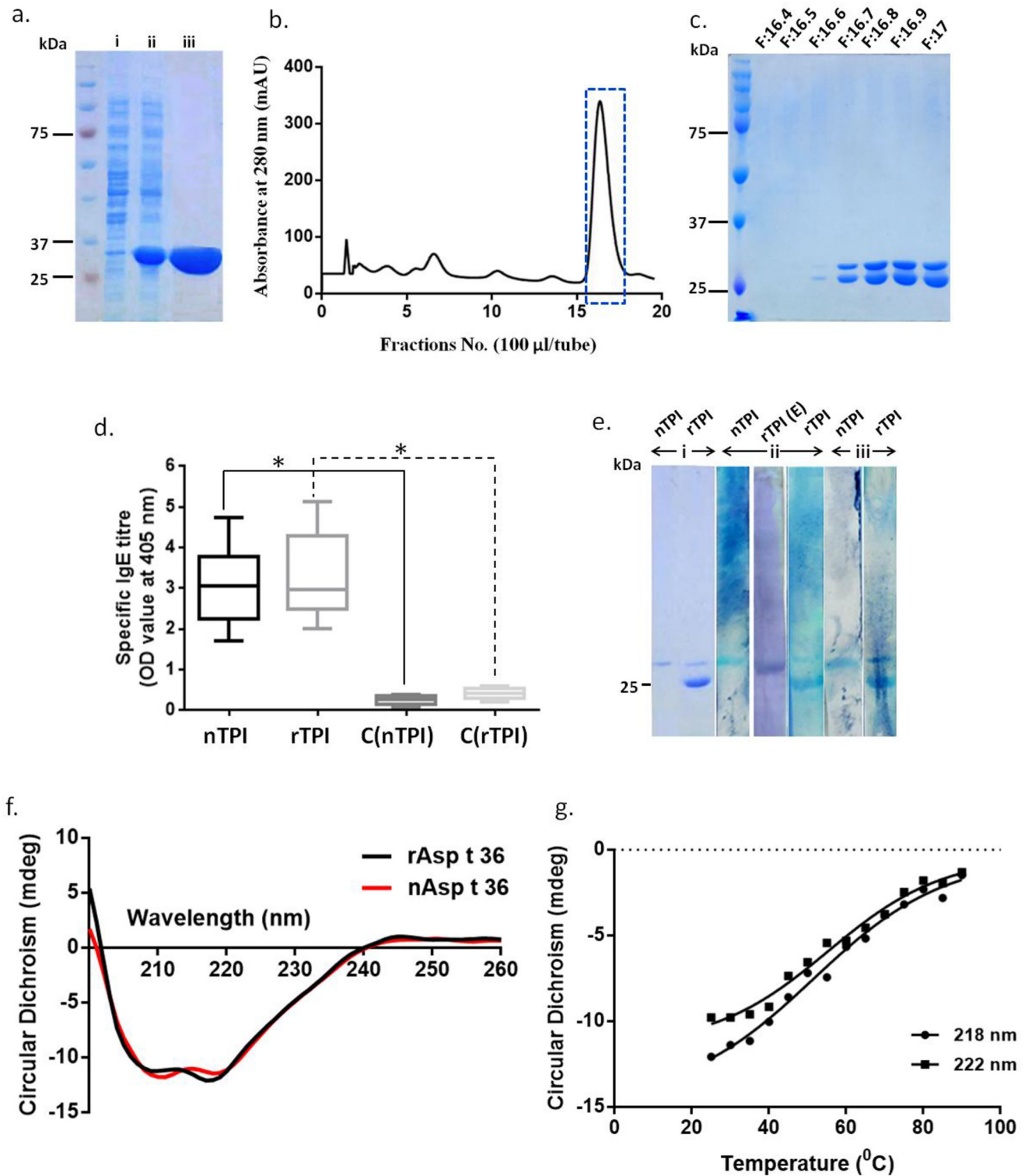


Figure 3. Expression, purification, and characterization of rTPI. *a*, SDS-PAGE of expressed rTPI in *E. coli*, (i) uninduced, (ii) induction with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside; desired protein found in the soluble fraction of the cell homogenate, (iii) after purification through nickel-nitrilotriacetic acid column. *b*, chromatogram from size exclusion chromatography of purified rTPI. *c*, SDS-PAGE profile of the collected protein fractions from gel filtration chromatography. The desired 28-kDa rTPI found to exist at 28 and 25 kDa (F:16.7-17). *d*, indirect ELISA of nTPI versus rTPI, performed with *A. terreus*-sensitized patients' sera ($n = 15$). Healthy patients' sera ($n = 3$) taken as control set. *e*, comparative study on the immunogenic specificity of nTPI and rTPI (i) SDS-PAGE of nTPI and rTPI, (ii) IgE blot with patients' serum pool ($n = 15$) against nTPI, induced *E. coli* extract, rTPI (E) and purified rTPI, (iii) IgG blot with rabbit anti-TPI polyclonal antibodies. *f*, comparative far-UV CD analysis between purified nAsp t 36 and rAsp t 36 at 25 $^{\circ}$ C from 200 to 260 nm. Both native and recombinant version of Asp t 36 showed similar secondary structure. *g*, the change in CD spectra of rAsp t 36 at 222 and 218 nm with increasing temperature from 25 to 90 $^{\circ}$ C. Sigmoidal curve fitting was done. C, control. Statistical analysis: one-way analysis of variance employing Tukey's multiple comparison test. * represents $p < 0.05$.

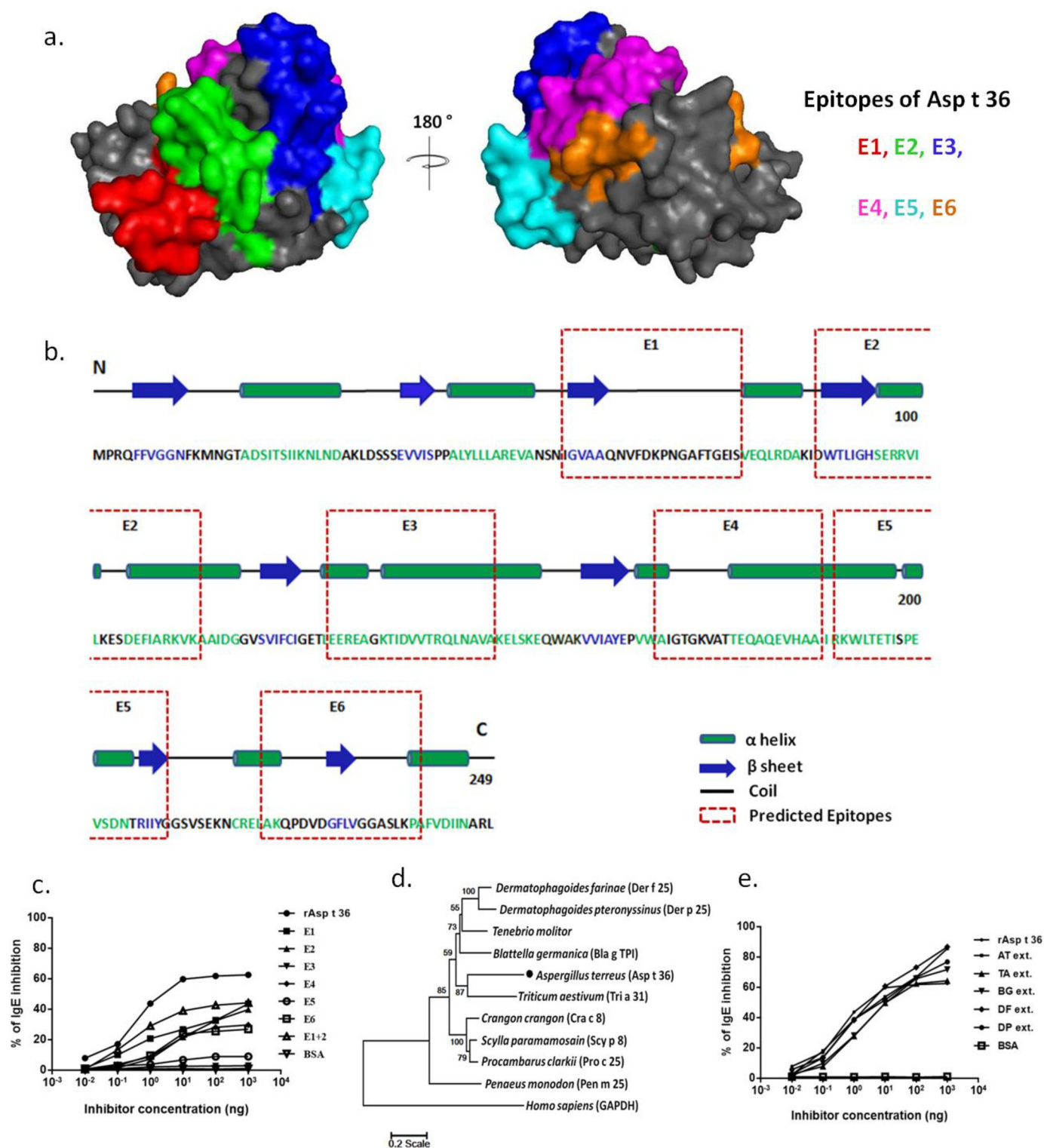


Figure 4. Structural modeling, epitope analysis, and phylogenetic relatedness of *A. terreus* Asp t 36 with other TPis. *a*, homology model structure of *A. terreus* Asp t 36 using TPI crystal structure from *T. molitor* as a template with six predicted epitopes (E1 to E6). *b*, schematic representation showing location of six linear epitopes on secondary structure of Asp t 36. Predicted linear epitopes are represented with red box. *c*, allergenic potency assessment of synthetic peptides through ELISA inhibition. Increasing concentrations of synthetic peptides (*x* axis) were preincubated with *A. terreus* positive patients' serum pools (*n* = 15). rAsp t 36 was used as positive control and BSA as negative control. *d*, dendrogram showing evolutionary relationship between Asp t 36 (●) of *A. terreus* and TPI proteins from others. Bootstrap resampling was 1000 replicates. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as negative control. *e*, cross-reactivity assay of Asp t 36 with phylogenetically related species having TPI as an allergen. In this assay, increasing concentrations of antigenic extracts (*x* axis) from four different species were preincubated with *A. terreus*-positive patients' serum pools (*n* = 15). *A. terreus* protein extract, rAsp t 36 was used as positive control and BSA as negative control. AT Ext., *A. terreus* protein extract; TA Ext., *T. aestivum* protein extract; BG Ext., *B. germanica* protein extract; DF Ext., *D. farinae* protein extract; DP Ext., *D. pteronyssinus* protein extract.

Asp t 36, a novel allergen from *A. terreus*

with the synthetic peptides using 15 individual patients' sera (Fig. S3, *a* and *b*). Among these peptides, E1, E2, E4, and E6 were found to trigger IgE response significantly higher than control. E1 and E2 were found to be closely spaced in the 3D map and could resemble an IgE-binding hotspot.

To analyze the relevance of IgE-binding ability of individual synthetic peptides (E1–E6), IgE ELISA inhibition experiments were performed using the pooled sera of *A. terreus* allergic patients (Fig. 4c). This led to partial inhibition of IgE binding to plate-bound rAsp t 36. 50% of IgE inhibition was achieved at 4 ng of rAsp t 36 as self-inhibitor. But none of the individual peptides (fluid phase), achieved the IC₅₀ (50% of IgE inhibition) value at 4 ng/ml. Individual peptides displayed ~23, 14, 3, 13, 5, and 16% IgE inhibition, respectively. At 4 ng/ml, the IgE inhibition value for E1 and E2 combined was ~32%. Hence, two adjacently placed epitopes, often acting as a conformational epitope on the 3D structure of a protein has a higher ability to bind with free IgE antibodies of the patients' sera.

Multiple sequence alignment and phylogenetic tree

On the basis of amino acid sequence alignment (Fig. S4), a phylogenetic tree was constructed between Asp t 36 and eight other TPI allergens along with TPI from *T. molitor* to determine their sequence conservation and evolutionary distance (Fig. 4d). The sequence of *A. terreus* Asp t 36 revealed 98.0% similarity to house dust mite (*Dermatophagoides pteronyssinus*; Der p 25), German cockroach (*Blattella germanica*; Bla g TPI), shrimp (*Crangon crangon*; Cra c 8), tiger shrimp (*Penaeus monodon*; Pen m 8), mud crab (*Scylla paramamosain*; Scy p 8), freshwater crayfish (*Procambarus clarkii*; Pro c 8), wheat (*Triticum aestivum*; Tri a 31), and 99% similarity to another house dust mite (*Dermatophagoides farinae*; Der f 25). Upon using a neighbor-joining method, the closest similarity was found with wheat based on evolutionary distance. However, the close similarity was also observed with German cockroach and *D. pteronyssinus*. All the crustaceans were present in a separate cluster and supposed to be more evolutionary diverged from the Asp t 36.

Cross-reactivity assessment of Asp t 36

Cross-reactivity is supposed to be mediated by the presence of similar allergens between two species that share common IgE-binding epitopes where they may have high sequence similarity. TPI being a highly conserved protein, we reasoned that Asp t 36 could be cross-reactive to other species. Cross-reactivity was checked with three aeroallergens and wheat based on phylogeny by ELISA inhibition. 98 ng of TPI from *T. aestivum*, 53 ng of TPI from *B. germanica*, 21 ng of TPI from *D. farinae*, and 32 ng of *D. pteronyssinus* was required for 50% inhibition of IgE binding to rAsp t 36 bound in the solid phase on the plates (Fig. 4e). 17 ng of self-antigen rAsp t 36 and 31 ng of antigenic crude extract from *A. terreus* was also found to show 50% IgE inhibition. On the basis of this result, the strongest cross-reactivity is observed mostly in *D. farinae*, which was also observed to be closely related in the phylogenetic analysis. Although they are present in the same cluster, cross-reactivity with wheat was much lesser compared with the other.

The airway inflammatory response and allergenicity assessment in rAsp t 36-challenged mice

To determine the role Asp t 36 on allergic airway inflammation, Balb/c mice were challenged intranasally with rAsp t 36, and allergic inflammatory parameters, such as eosinophilic inflammation and mucus secretion in lung tissue were analyzed (Fig. 5, *a–e*). In H&E staining, the lung sections revealed the highest increment of eosinophil infiltration ($p < 0.01$) in the peribronchial spaces. Mucus-secreting goblet cell proliferation inside the bronchial basement membrane was found to be higher in the rAsp t 36-challenged mice ($p < 0.05$) as compared with the OVA-challenged mice using periodic acid–Schiff (PAS) staining. However, PBS-challenged mice exhibited the lowest airway inflammatory responses in both cases of histopathological studies. The antibody response in three groups of mice was measured from sera. The rAsp t 36-challenge led to induction of rAsp t 36-specific IgE, IgG1, IgG2a/2b, and their level was significantly ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) higher compared with PBS-challenged mice (Fig. 5, *f–h*). In OVA-challenged mice, IgE titer was also increased, but their level was low compared with rAsp t 36-challenged mice. The cytokine profiling in three groups of mice sera revealed that IL-4 and IL-5 levels were increased significantly ($p < 0.05$, $p < 0.01$) in rAsp t 36-challenged mice than PBS-treated mice. The level of IL-13 in both rAsp t 36 and OVA-challenged mice was almost same but higher than the control group (both $p < 0.05$). In the case of IFN- γ , its level was decreased in rAsp t 36-challenged mice compared with OVA-challenged subjects (Fig. 5, *i–l*). In the rAsp t 36-sensitized rabbit, there was a strong IgG response after immunization. The IgG levels in the sera of rAsp t 36-immunized rabbit were significantly higher ($p < 0.05$) than the preimmunized rabbit sera (Fig. S5).

Discussion

Aspergillus species have long been identified as a potent source of respiratory maladies. A number of them have also been reported as allergenic, and efforts were made to identify the causative proteins. The present study describes the identification of allergens from *A. terreus*, one of the dominant aeromycoflora from eastern India (2). *A. terreus* is well-known as a plant pathogen affecting a wide range of crops (16). It is also known to cause opportunistic infections in immunocompromised patients as well as superficial lesions (17). Its identity as an allergenic fungus is reported here for the first time. Identifying the allergens and characterizing IgE-binding epitopes on them assists in understanding the molecular basis of allergenicity. Moreover, the identification of conserved epitopes could help in developing potent vaccines that could target a wide spectrum of allergenic sources harboring similar allergens. This study has not only delineated the allergens present in this novel fungus but also gave initial insights into the IgE-binding epitopes on one of the allergens, triose-phosphate isomerase.

An immunoproteomics approach was used to identify the IgE reactive proteins from *A. terreus*. The identified allergens can be categorized into two groups. One group of proteins belongs to the already known allergens from fungal/other

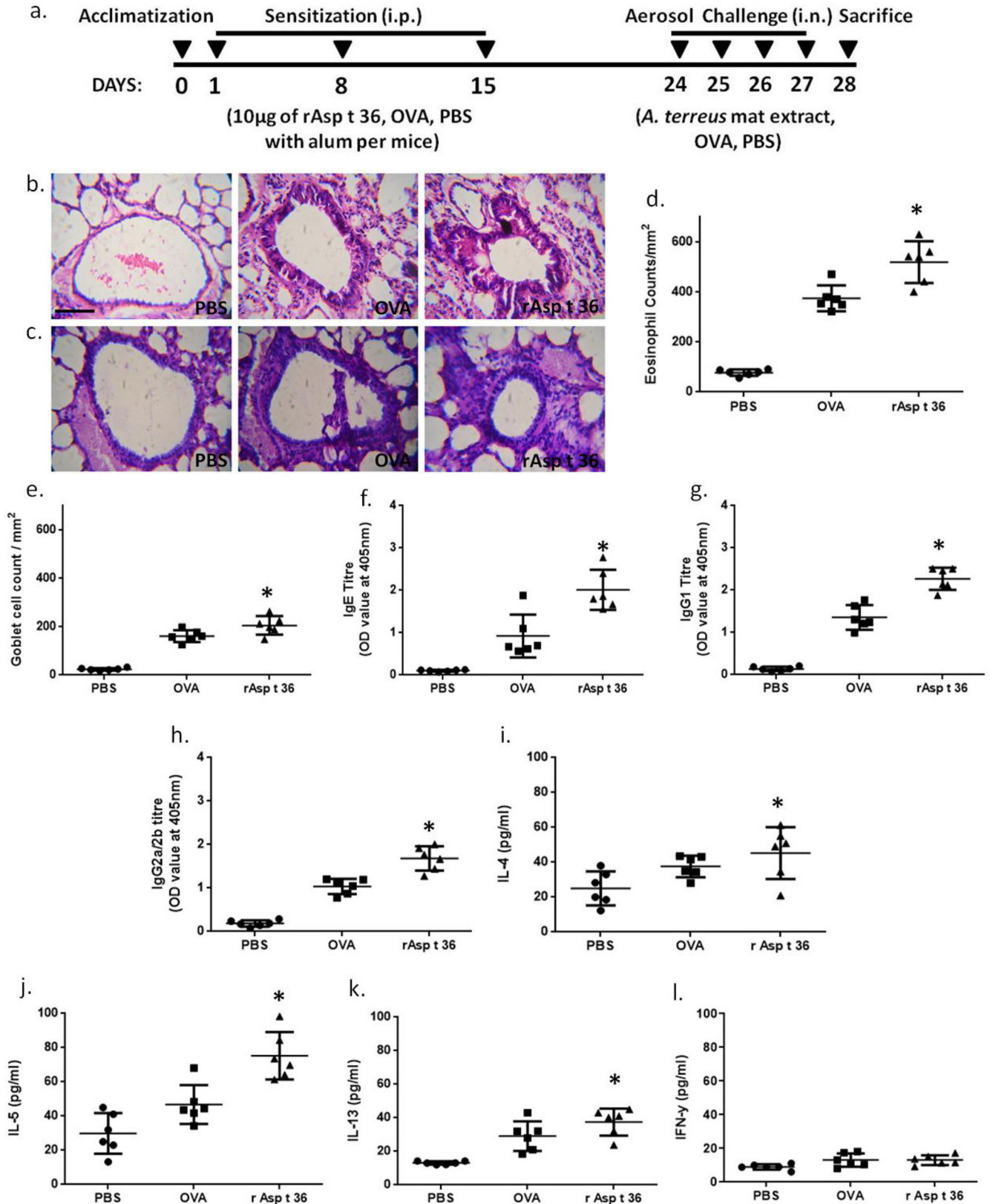


Figure 5. Airway inflammatory response and allergenicity assessment in a rAsp t 36-challenged BALB/c mice model. *a*, experimental design of allergen challenge in three groups of mice ($n = 6/\text{group}$); experimental group, rAsp t 36; positive control, ovalbumin; negative control, PBS. *b* and *c*, mice lung histology with H&E-stained tissue and PAS-stained tissue. *d* and *e*, scatter plot showing eosinophil counts in spaces of lung and goblet cell counts in bronchial basement membrane of lung. *f-h*, analysis of Ig subclasses titer (IgE, IgG1, and IgG2a/2b) in the serum of mice treated with ELISA. *i-l*, scatter plot shows cytokines profile in the serum of treated mice. Statistical analysis: one-way analysis of variance employing Tukey's multiple comparison test. * represents $p < 0.05$ in rAsp t 36-challenged versus PBS-treated mice. *i.p.*, intraperitoneal; *i.n.*, intranasal. Scale bar: 100 µm.

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species, and the other includes completely novel allergens. Among 16 immunoreactive proteins: glutamine synthetase, phosphoglycerate kinase, fructose-bisphosphate aldolase, triose-phosphate isomerase, and GSH-S-transferase had already been enlisted in allergen category. Phosphoglycerate kinase, fructose-bisphosphate aldolase, GSH-S-transferase were reported as allergens from fungi such as *Candida albicans*, *Rhizopus oryzae*, and *Alternaria alternata* (6, 18, 19). Glutamine synthetase from *Cannabis sativa* has also been accounted for showing allergenicity (18). The proteins identified as hypothetical or predicted were searched for similarity in the NCBI database using BLASTp. This revealed spots 4, 15, 17, and 18 to have similarity with semi-aldehyde dehydrogenase: NAD-binding (*Penicillium expansum*), GSH-S-transferase (*Penicillium subrubescens*), cysteine-rich protein (*Metarhizium acridum*), and ADP-ribosyltransferase exoenzyme (*Actinorhizopora endophytica*), respectively. Immunoblotting with individual patients' sera revealed spots 6, 13, and 17 to be reactive to more than 80% of the patients, whereas spots 14, 15, and 18 were reactive to less than 40% of the patients suggesting them to be minor allergens. A disparity of molecular weights between observed and theoretical was noticed for spots 2, 7, 8, 14, 16, and 17. This could be because many proteins undergo different post-transcriptional modifications, may differ in structural subunits for proper functioning, and even sometimes, their expression depends on environmental stress conditions (20).

Among the allergens, triose-phosphate isomerase was found to be a common cause of food allergy in crustaceans, molluscs, and arthropods (15, 21). It has never been reported in any fungi, although some evidence was found in plants (22). Interestingly, all patients in our study cohort were found to be immunoreactive to this protein in the 2D blot and 1D SDS-PAGE blot suggesting it to be a major contributor to IgE sensitization. We were then interested in characterizing this protein further and investigating its cross-reactivity with established sources of this allergen.

As a first step, Asp t 36 was purified under native conditions using serial chromatographic methods. Profiling the nAsp t 36 in a broad range 2D gel, a single protein spot was observed in the desired region suggesting its high purity. Asp t 36 was also overexpressed in *E. coli*, and to check the presence of any contaminants, the purified protein was analyzed by MALDI for intact mass, which showed a major monomeric population of 28 kDa along with a small dimeric population of 56 kDa (Fig. S6). Although most TPI allergens were found to exist as a monomer, similar to our findings, few studies also revealed the existence of this protein as a dimer (21, 23). To validate the stoichiometry, rAsp t 36 eluted at the monomeric region in the size exclusion chromatogram. However, in the SDS-PAGE, rAsp t 36 revealed two bands of 28 and 25 kDa. Interestingly, both bands were identified as triose-phosphate isomerase. It could be hypothesized that there must be a defined cleavage site that is somehow prone to proteolytic degradation, giving rise to two different protein bands. The reason for this cleavage was, however, not investigated thoroughly in this study. High IgE titers evoked by both nAsp t 36 and rAsp t 36 revealed that they are immunologically active for further study.

Solubility, stability, and the compactness of the overall fold are likely to be relevant for allergenicity of a protein (24, 25). Asp t 36 was found to be relatively stable in mild temperature conditions and temperature-induced refolding was not possible as revealed by CD analysis. Far-UV CD spectra of TPI from *P. monodon* showed a similar temperature sensitivity profile (26). Moreover, having similar secondary folds in both the version of the protein, rAsp t 36 shares the same biological and immunological properties as its native counterpart. So, rAsp t 36 can be used as an alternative to the natural protein for diagnostic purposes and molecular investigations.

The 3D structure of other known TPis is a conserved ($\beta\alpha$)₈ barrel motif, composed of 8 central β -strands surrounded by 12 α -helices joined by loops (14). Asp t 36 also displayed a similar structure based on homology modeling. Understanding the structural features of allergens provides insights into their functions and the extent of IgE binding. IgE-binding epitopes could be linear or conformational. TPI being mostly represented as a food allergen may have linear epitopes that play a dominating role in determining atopicity (21). Linear epitopes represent stretches of 8-15 amino acids that lie in the exposed region of an allergen to facilitate antibody binding. In this study, we focused only on the linear epitopes. Six predicted IgE-binding epitopes were mapped on the structure of Asp t 36 (Fig. 4a). To further validate these predictions, ELISA inhibition was done to determine the potency of each epitope to inhibit IgE binding by preincubating sera with an increasing dosage of synthetic peptides (Fig. 4c). Epitopes were predicted recently on Der f 25, a TPI protein from house dust mite. It was found that there was significant sequence homology between predicted epitopes of Der f 25 and three peptides from Asp t 36: E1, E3, and E4 (27). E1, E2, E4, and E6, four immunodominant epitopes face mainly β -sheet and coil regions of the protein. Although E1 and E2 were synthesized as two different peptides, in the tertiary fold, these two coiled regions created a continuous patch of IgE-binding hotspot that may be capable of cross-linking and recruiting mast cells for degranulation. E1:E2 jointly demonstrated higher inhibition of preimmunized sera compared with individual peptides suggesting that these could be used in the design of hypoallergenic variants. A similar observation is found in aspartyl endopeptidase Rhi o 1 from *R. oryzae* where the C- and N-terminal region generated a hotspot for allergen binding (28).

The primary sequence of Asp t 36 is highly conserved across species despite some minor variability. A phylogenetic tree, built using Asp t 36 with eight other TPI allergens, revealed similarity with TPI from wheat, German cockroach, and house dust mite. The high sequence homology leads to the formation of similar folds, thus conserving the IgE-binding epitopes across species. This causes a significant level of cross-reactivity with other phylogenetically related or unrelated species (29). Der f 25 caused wide cross-reactivity due to its conserved structural features with different species (30). Similarly, TPI allergen in German cockroach was found to be cross-reactive between arthropods and other invertebrates (31). The difference in binding activity between *A. terreus* and *T. aestivum* was observed, suggesting that some of the immune epitopes might also vary.

Indeed, the Asp t 36 had 99% amino acid sequence homology with Der f 25 based on multiple sequence alignment and was found to be the most cross-reactive with *D. farinae* protein extract. Studies on these cross-reactive allergens may help maximize the efficiency of diagnosis of *A. terreus* allergic disease and minimize the components required for component-based immunotherapy in the future.

A fungal allergen-induced mice model was developed to investigate whether aero-allergen exposure would affect the secondary immunoinflammatory responses in the airways. Upon challenge with rAsp t 36, mouse lungs exhibited characteristics of the chronic phase of airway tissue remodeling. Both OVA and rAsp t 36 challenged mouse-induced peribronchial invasion of inflammatory cells and mucous hyperplasia. Increased depositions of collagen surrounding the bronchi combined with mucous hyperplasia are a typical response to the inhalation of foreign bodies (32). Representative H&E-stained and PAS-stained sections revealed Asp t 36 plays a critical role in initiating and promoting respiratory inflammation and allergy. High titers of rAsp t 36-specific IgE, IgG1, and IgG2a/2b ensure that the test animals have been exposed to a sufficient amount of allergic protein to induce an immune response. The increased expression of Th2 cytokines IL-4 and IL-5 by airway inflammatory cells observed in our animal model is in agreement with previous experimental and clinical studies, which shows that these cytokines contribute either directly or indirectly to promoting the differentiation, survival, and function of key allergic effector cells (33). The level of IL-13, the presumed central mediator of murine allergic airway inflammation was the same either by OVA sensitization or after rAsp t 36 challenges (34, 35). Conversely, IFN- γ being a cytokine that is released due to Th1 responses did not show any significant change after allergen challenge compared with control. This suggests that Asp t 36 induces a strong Th2 response, a hallmark of allergic pathophysiology, thus validating its allergenic properties. The ability to generate an IgG antibody response may be a result of the immunogenicity of the induced allergen protein in the rabbit strain of choice or may relate to prior exposure to a cross-reactive protein (36).

This study revealed a detailed molecular characterization of Asp t 36, a 28-kDa cross-reactive allergen from *A. terreus*. Its allergenicity in an animal model reinforces its strong atopic response and can be used for further *in vivo* studies. Thus, Asp t 36 may be a useful candidate to be explored for its potential role in the differential diagnosis of *A. terreus*-associated allergic diseases and immunotherapy purposes.

Experimental procedures

Clinical study, patient selection, and sera collection

Patients suffering from allergic rhinitis and asthma with a consistent family history of allergy were selected as study subjects from the total patients' pool who visited Habra State General Hospitals, Habra, India. 10 ml of venous blood were collected in heparinized vial from 15 *A. terreus*-sensitized patients based on skin prick testing and three nonatopic patients with their written consent. The study was approved by the Bose Institute Human Ethics Committee (reference number BIHEC/

2014–15/4) following the declaration of Helsinki principles. After 30 min, sera were separated by spinning the blood sample at 1000 rpm for 10 min at 4 °C and stored at 4 °C for immediate use or at –20 °C for long term usage.

Extraction of crude protein

A. terreus was cultured in potato dextrose agar media (PD, 20 g/liter; A, 13 g/liter) for 2 weeks at 30 °C. The lyophilized fungal mat harvested at their sporulating phase was taken for protein extraction following the Tris-phenol protein extraction protocol, as previously described (6). In brief, the protein was pelleted from the phenol phase by adding 5 volumes of 0.1 M ammonium acetate in chilled methanol. After washing the pellet twice in methanolic ammonium acetate and chilled acetone, the air-dried pellet resuspended in 50 μ l of rehydration solution was assayed for protein concentration using Bradford reagent.

Estimation of specific IgE

The microtiter plates (Nunc, Thermo, USA) were coated with 1 μ g of fungal protein extract overnight at 4 °C (37). After blocking with 1% BSA, the plate was incubated for 16 h at 4 °C with individual *A. terreus*-sensitized patients' sera at a 1:10 (v/v) dilution. Secondary antibody incubation was done with monoclonal anti-human IgE alkaline phosphatase conjugate (Sigma, USA) for 3 h at room temperature (1:1000 v/v). para-Nitrophenylphosphate (Thermo Scientific) was used as substrate and absorbance was measured at 405 nm (Multiskan-Labsystem, Finland).

2D gel profiling of *A. terreus*

160 μ g of total protein in rehydration buffer was passively soaked overnight in 4–7 pI IPG strips (GE Healthcare). Isoelectric focusing was done in the Ettan IPG Phor system (GE Life Sciences) according to the manufacturer's protocol. Strips were then reduced in equilibration buffer I and alkylated in buffer II. Finally, strips were laid on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue R250. 2D gels images were analyzed in ImageMaster 2D Platinum for pI and molecular weights.

Western and dot blotting

For Western blotting, the 2D gels were electrotransferred onto the high bond LFT polyvinylidene difluoride membrane (GE Life Sciences) using a semi-dry method. After blocking membranes using 3% BSA, it was incubated with primary and secondary antibody as described previously to estimate specific IgE. IgE reactive spots were observed by adding nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) substrate solution. 2D Western blotting was done using both pooled sera and individual sera. For dot blotting, nitrocellulose membranes (GE Life Sciences) cut in 3-mm radial discs was dotted with 4 μ l of protein and assayed similarly.

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Identification of allergens by MS

Sample preparation for identifying proteins through MS was done as previously described (38). Protein spots were excised from the gel and destained with 50 mM ammonium bicarbonate (ABC) and ethanol (1:1, v/v) followed by dehydration in acetonitrile. Thereafter, spots were reduced and alkylated in 10 mM DTT and 55 mM iodoacetamide, respectively. After hydrating and dehydrating alternatively in ABC and acetonitrile, protein spots were in-gel digested overnight at 37 °C with 12.5 ng/μl of trypsin (Promega, USA). Peptides were extracted in 3% TFA with 30% acetonitrile, vacuum dried, and reconstituted in 5 μl of 0.1% TFA. Equal volumes of peptides and α-cyano-4-hydroxycinnamic acid matrix were mixed. 2.5 μl of the mixture was spotted onto an MTP 384 ground steel target plate (Bruker Daltonics) in duplicates and air-dried. Spectra were acquired on Autoflex II MALDI-TOF/TOF (Bruker Daltonics, Germany). Peptide peaks with high signal to noise ratio (>15) were selected manually and fragmented by collision-induced dissociation. The parent and the product ions were analyzed using the SNAP algorithm in Flex analysis, version 3.4 (Bruker Daltonics). Baseline subtraction and smoothing of mass spectra were done in the flex analysis software. The processed peaks were transferred to biotools 3.2 (Bruker Daltonics), which inputs data into MASCOT search engine version 2.4.1 (RRID:SCR_014322) for protein identification. The following parameters were maintained: mass tolerance of parent ion, 1.2 Da; fragment ion, 0.5 Da; variable modification, methionine oxidation; fixed modification, carbamidomethylation of cysteine; taxonomy, Fungi; database, *A. terreus* in NCBI against 55320 proteins as on 24.08.2017. Protein identification was considered reliable only if they were beyond significance ($p < 0.05$) and at least two unique peptides have ion scores beyond the threshold limit assigned. However, trypsin autolysis and keratin peaks were removed by the internal calibration of the instrument. A maximum of two missed cleavages was permitted.

Purification of IgE reactive TPI

Total fungal protein was extracted in binding buffer (20 mM Tris-HCl, pH 7) and loaded onto a strong anion exchanger Q HP (GE Life Sciences). Bound proteins were eluted with 1 M NaCl gradient in binding buffer. Desired protein fractions were further purified by passing sequentially through 10- and 30-kDa centrifugal filters (Amicon, Millipore USA) to remove other contaminating proteins. TPI was further purified and polished by size exclusion chromatography (Superdex 200 increase 10/300 GL, GE Life Sciences) in 0.01 M phosphate buffer, 0.14 M NaCl, pH 7.4.

Cloning and overexpression of recombinant TPI

Total *A. terreus* RNA was extracted from the fungal mat with TRIzol (Thermo Fisher, USA) according to the manufacturer's protocol. Amplification of full-length cDNA, generated using iScript cDNA synthesis kit (Bio-Rad, USA) was achieved by using primers (forward, 5'-AATTGGATCCATGCCTCGC-CAATTCTTCG-3', and reverse, 5'-ATAACTCGAGTTACAGACGAGCGTTGATGATG-3') having BamHI (5') and XhoI

(3') restriction sites designed from the nucleotide sequences reported in NCBI. The rTPI amplicon was directionally cloned into the pET28a vector containing N-terminal His tag and transformed into *E. coli* BL21 strain for protein expression. Protein was purified using nickel-nitrilotriacetic acid column (Qia-gen) and gel filtration from the soluble fraction of the lysed cells.

CD

Far-UV circular dichroism (CD) spectra, recorded from 200 to 260 nm were acquired at a regular interval of 5 °C from 25-90 °C using CD spectro-polarimeter (Jasco Corp. J-815, Japan) for nAsp t 36 and rAsp t 36 at a concentration of 0.15 mg/ml. The sample was reconstituted in 0.01 M phosphate buffer and analyzed in a 1-mm optical path length cell. The results were expressed as the mean residue expressed in ellipticities (degree cm²/dmol). Finally, CD spectra were analyzed through the DICHROWEB server. Temperature-induced unfolding of Asp t 36 was monitored for α-helix and β-sheet by recording CD values at 222 and 218 nm, respectively. Melting temperature was determined after performing a sigmoidal curve fitting. Data were smoothened using Prism Software.

In silico study: homology modeling, epitope prediction, their synthesis and IgE-binding assay

The homology-based model of Asp t 36 was generated by Swiss Model using the crystal structure of TPI from *T. molitor* as a template, showing maximum sequence identity in the PDB database. Stereochemical quality was checked in the SAVES (version 5) server, and PROCHECK. Linear IgE epitopes were predicted using online B cell epitope prediction tools: ABCPred, BCPred, BCEPred, and BepiPred 1.0b server. The epitopes were synthesized in Endeavor 90-II peptide synthesizer (AAPPTec, Louisville, KY, USA) according to established protocols (39). Finally peptide purified by HPLC was validated by MALDI-TOF. For the IgE-binding assay of the peptides, dot blot and ELISA were performed with 100 μg of each peptide.

Sequence homology search in database

Allergens similar to Asp t 36 were searched in the SDAP and WHO/IUIS database for generating a phylogenetic tree. The sequence similarity search performed in the CLUSTAL omega software using the entire amino acid sequence of the Asp t 36 with reported TPI allergens from eight species. The phylogenetic tree was constructed using Mega 7.0.

ELISA inhibition assay

A. terreus-positive patients' serum pools were preincubated with 10-fold serial dilution of *A. terreus* protein extract, antigenic extracts (All Cure Pharma, New Delhi, India) from four different species or synthetic peptides. This serum pool was then loaded on the well-coated with 100 ng/μl of *A. terreus* rAsp t 36 and the ELISA reading was collected as described above. Percentage inhibition was calculated by Equation 1.

$$1 - \frac{\text{OD}_{405} \text{ of sample with inhibitor}}{\text{OD}_{405} \text{ of sample without inhibitor}} \times 100 \quad (\text{Eq. 1})$$

Animal model study, measurements of airway inflammation, and immune responses in Asp t 36-sensitized mice

BALB/c male mice (6–8 weeks of age) from three groups were immunized intraperitoneally (10 µg/mice) with rAsp t 36 (experimental group) or ovalbumin (positive control) or PBS (negative control) following the protocol proposed by Groome *et al.* (33). For the development of antisera, one New Zealand White rabbit (200 µg of antigen/rabbit) and three mice groups (10 µg of antigen/mice) were immunized with rAsp t 36 adsorbed to Freund's adjuvants following a standard protocol (40). All experiments were approved by the Bose Institute ethics committee for animal welfare (reference number IAEC/BI/41/2015). For mice, blood was collected by cardiac puncture on the final day of the protocol. After 15 days of the third booster dose, blood was drawn from the rabbit in a 5-ml heparinized sodium vial. Sera were separated by centrifuging at 1000 rpm for 10 min storage at 4 °C for immediate use or at –20 °C for long term usage. For histopathological analysis, the lungs of mice were stained by the H&E and PAS staining according to standard protocols (32). For the detection of Asp t 36-specific mice, IgE, IgG1, IgG2a/2b, and rabbit IgG, microtiter plates (Nunc, Thermo, USA) were coated overnight with 5 µg/ml of rAsp t 36. Serum was diluted 1:100 for mice IgE, 1:10,000 for mice IgG1, IgG2a/2b, and rabbit IgG. The preimmune sera from rabbit were used in parallel as controls. ELISAs were performed by using a kit from BD Pharmingen, San Diego, CA, USA, and Sigma-Aldrich. The remainder of the protocol was carried out according to the manufacturer's instructions. Inflammatory Th2 blood cytokine levels such as IL4, IL5, and IL13 and anti-inflammatory IFN-γ were detected using ELISA kits (Sigma) following the manufacturer's protocol.

Statistical analysis

Statistical analysis on the comparative study of inflammation in lung histology, antibody, and cytokine level between rAsp t 36-challenged and PBS-challenged mice was done by using GraphPad Prism software (GraphPad, San Diego, CA, USA; version 6).

Data availability

All data described are available in the manuscript and associated supporting information file. Allergen submissions data are available on the WHO/IUIS allergen nomenclature database (www.allergen.org/viewallergen.php?aid=998). The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PDX021124.

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Abbreviations—The abbreviations used are: Asp, alkaline serine proteinase; TPI, triose-phosphate isomerase; PAS, Periodic acid–Schiff; aa, amino acid(s); ABC, ammonium bicarbonate; IL, interleukin; IFN, interferon; nTPI, native TPI; rTPI, recombinant TPI.

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Identification and biochemical characterisation of Asp t 36, a new fungal allergen from *Aspergillus terreus*

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List of the material included:

Table S1. Demographic details of patients showing allergic sensitivity to *Aspergillus terreus*.

Table S2. Identification of allergenic proteins through MALDI-TOF/TOF from *A. terreus*.

Figure S1. Serological screening for allergenicity determination with the total proteome of *A. terreus*.

Figure S2. Mass spectra of the purified native TPI from *A. terreus* using MALDI TOF/TOF. The sequences of each peptide provided.

Figure S3. Immunogenicity assays with six different synthetic peptides of Asp t 36.

Figure S4. Multiple sequence alignment of amino acid sequences of Asp t 36 from *A. terreus* and phylogenetically related TPI from other species.

Figure S5. A comparative study on IgG level before and after immunization of rabbit with Asp t 36.

Figure S6. Mass spectra of the intact recombinant protein (rAsp t 36), purified through Ni-NTA column using MALDI TOF/TOF.

Tables:

Table S1. Demographic details of patients showing allergic sensitivity to *Aspergillus terreus*.

Patient Number (*)	Sex (*)	Age	SPT Gradation (#)	Specific IgE value	Clinical History (**)
P1	M	41	3 +	3.6	AR, BA
P2	F	38	3 +	4.5	BA, CC
P3	F	54	3 +	4.9	AR, SR
P4	M	26	2 +	3.6	AR, CC
P5	F	19	2 +	2.8	AR, BA,
P6	M	59	3 +	3.5	AR
P7	M	42	2 +	3.2	AR, BA
P8	M	39	2 +	3.1	AR, BA, CC
P9	M	22	3 +	3.1	AR, SR
P10	M	25	1 +	3.4	AR, SR
P11	F	33	2 +	2.4	AR, CC
P12	F	27	3 +	4.4	AR
P13	M	49	1 +	2.3	AR, BA
P14	M	31	3 +	3	AR
P15	F	29	1 +	2	AR
C1	M	26	-	0.5	NA
C2	M	29	-	0.4	NA
C3	F	56	-	0.5	NA

*Abbreviations: P: Patient, M: Male, F: Female, C: Control. (Patients having smoking habits, drug addiction, and/ other chronic diseases were not incorporated in the study).

**Abbreviations: AR: Allergic rhinitis, BA: Bronchial asthma, CC: Cough & cold, SR: Skin rash, NA: Non atopic.

SPT Gradation: 1+: No wheal, erythema diameter < 20mm; 2+: No wheal, erythema diameter > 20 mm and 3+: Wheal and erythema. SPT: Skin prick test.

9.	11.	Formate dehydrogenase	<i>Aspergillus terreus</i> NIH2624	EAU33446.1	MVFLR MVFLRSFSR VLMVLYDGGEHAK QQPGLLGTTELGLR EGENSTFDKELVDAEVIITTPFHPGYLTAER ELVDAEVIITTPFHPGYLTAER NFVPAHEQIR IGERVLR ELLYYDYQPLKPEIEK EIGCR RVEDLEEMLAQCDVVTINCPLEK GYGGDVWFPQAPK YVQGPWGGGNAMVPHMSGTSIDAQIR HDYRPEDLIVK QQPGLLGTTELGLR (1725.9620) ELVDAEVIITTPFHPGYLTAER (2471.323) RVEDLEEMLAQCDVVTINCPLEK.T (2897.4640)	46/8.14	41/6.8	89 54 96 83	43% 3% 5% 5%	MS MS/MS MS/MS MS/MS
10.	12.	Formate dehydrogenase	<i>Aspergillus terreus</i> NIH2624	EAU33446.1	QQPGLLGTTELGLR EGENSTFDKELVDAEVIITTPFHPGYLTAER ELVDAEVIITTPFHPGYLTAER NFVPAHEQIR IGERVLR ELLYYDYQPLKPEIEK EIGCR RVEDLEEMLAQCDVVTINCPLEK EDVAAEAVK GYGGDVWFPQAPK YVQGPWGGGNAMVPHMSGTSIDAQIR AILESYFSGR HDYRPEDLIVK ELVDAEVIITTPFHPGYLTAER (2471.2940)	46/8.14	41/6.7	66 75	42% 5%	MS MS/MS
11.	13.	Triosephosphate isomerase # *	<i>Aspergillus terreus</i> NIH2624	EAU33924.1	LDSSEVVISPPALYLLAR EVANSNIGVAAQNVFDKPNGAFTGEISVEQLR IDWTLIGHSER RVILK AAIDGGVSVIFCIGETLEER TIDVVTR VATTEQAQEVHAAIR WLTETISPEVSDNTR IDWTLIGHSER (1326.6195) LDSSEVVISPPALYLLAR (2143.1021) EVANSNIGVAAQNVFDKPNGAFTGEISVEQLR (3375.5246)	27/5.42	28/5.6	75 84 111 118	50% 4% 8% 12%	MS MS/ MS MS/ MS MS/ MS
12.	14.	Heat shock protein 82	<i>Aspergillus terreus</i> NIH2624	EAU31169.1	DLEESEEEKAER (1739.3660) GVVDSIDLPLNLSR (1513.4270)	79/4.97	26/5.5	103 55	1% 1%	MS/ MS MS/ MS
13.	15.	Hypothetical protein ATEG_08454 #	<i>Aspergillus terreus</i> NIH2624	EAU31627.1	LLPIKVGQGGPNPPR VWQGGPNPPR EPEYLAINPNGR LPAIYDPNTDLTLWESGAIVEYLV YDTAHDISFPR QWLFFQASGQGPYYGQACWFK KYHPEPVPSALER YHPEPVPSALER CSFADLAWVSWQMTLK KIIQTEDGYDVENFPHLK IIQTEDGYDVENFPHLK NWLDR NWLDRMVA	26/5.61	26/5.62	78	62%	MS
14.	16.	S-(hydroxymethyl) glutathione dehydrogenase	<i>Aspergillus terreus</i> NIH2624	EAU34016.1	IRATQGR GVMPDGTTR EEWAYK EEWAYK FGATHFVNPAK GCAFGGIK GCAFGGIKGR MREGGCIIR CVVDTQR	33/8.64	24/6.6	54	19%	MS
15.	17.	Hypothetical protein ATEG_02590	<i>Aspergillus terreus</i> NIH2624	EAU37552.1	TGANAGQWLNDAR (1373.7262) FQLCADENCTANAPVNPGLVYIR (2709.3847)	38/5.78	19/5.62	64 47	3% 6%	MS/MS MS/MS

16.	18.	Predicted protein	<i>Aspergillus terreus</i> NIH2624	EAU37951.1	MADLPEFSR MADLPEFSR ADLPEFSR ERSDNIK LLLPFEL	11/5.61	11/5.7	39	21%	MS
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(The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021124).

Figures:

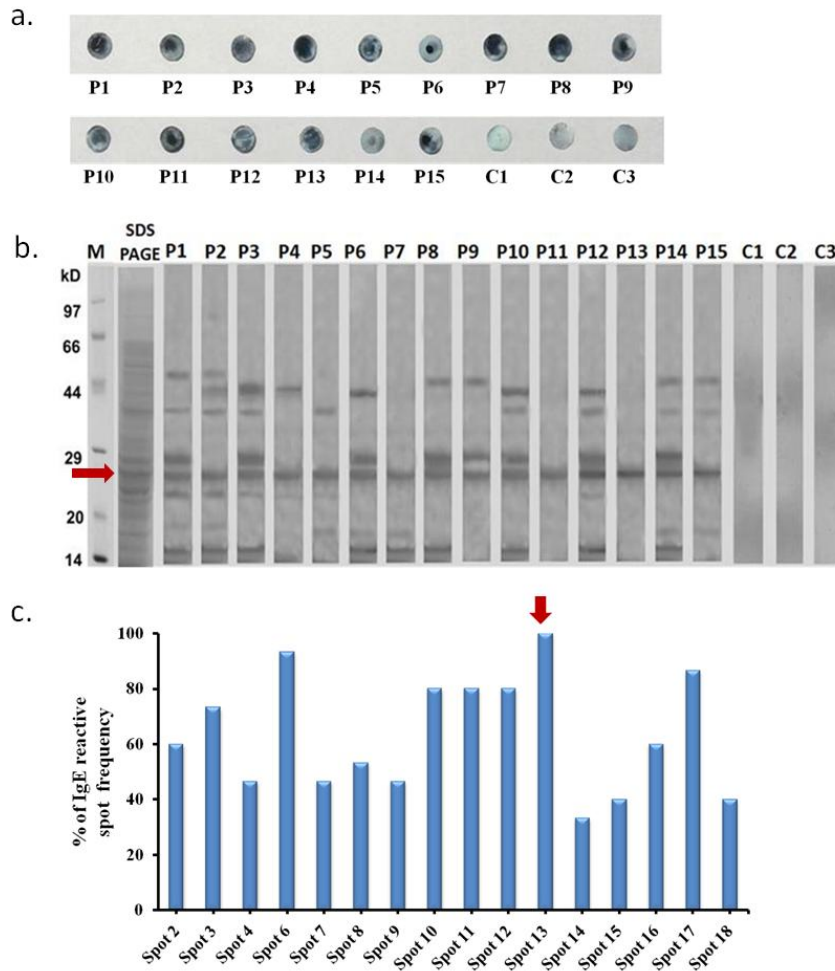


Figure S1. Serological screening for allergenicity determination with the total proteome of *A. terreus*. **a.** Dot blot results using fifteen *A. terreus* sensitized patients' sera and three healthy patients' sera. Relative dot blot spot intensities depend on corresponding SPT data per patient. **b.** Total protein profile on 12% SDS-PAGE, following 1D blot with individual patients' sera (n=15) and control sera (n=3). The 28kDa protein band was found to be IgE reactive for most of the sensitive patients. **c.** Bar graph showing the percentage of IgE reactive protein spots frequency, followed by the determination of major allergen, 28kDa (Spot 13). Arrows point out the 28kDa protein bands and major allergen. P1 to P15: Patients, C1, C2, C3: Control.

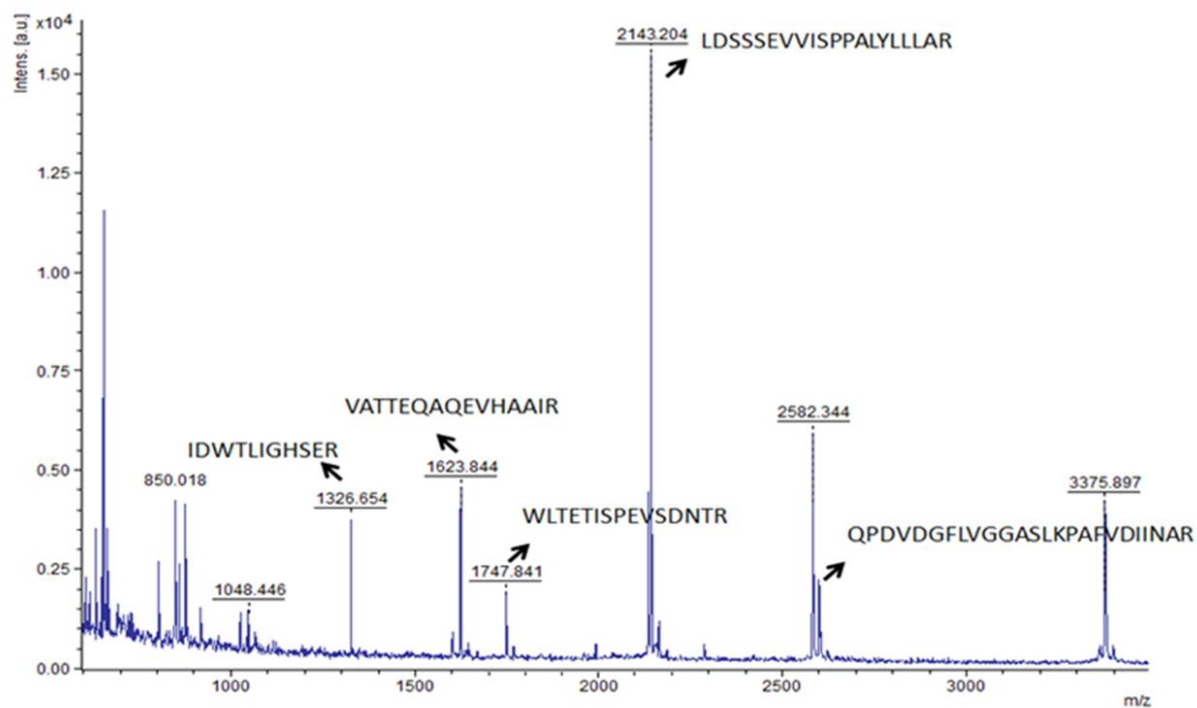


Figure S2. Mass spectra of the purified native TPI from *A. terreus* using MALDI TOF/TOF. The sequences of each peptide provided.

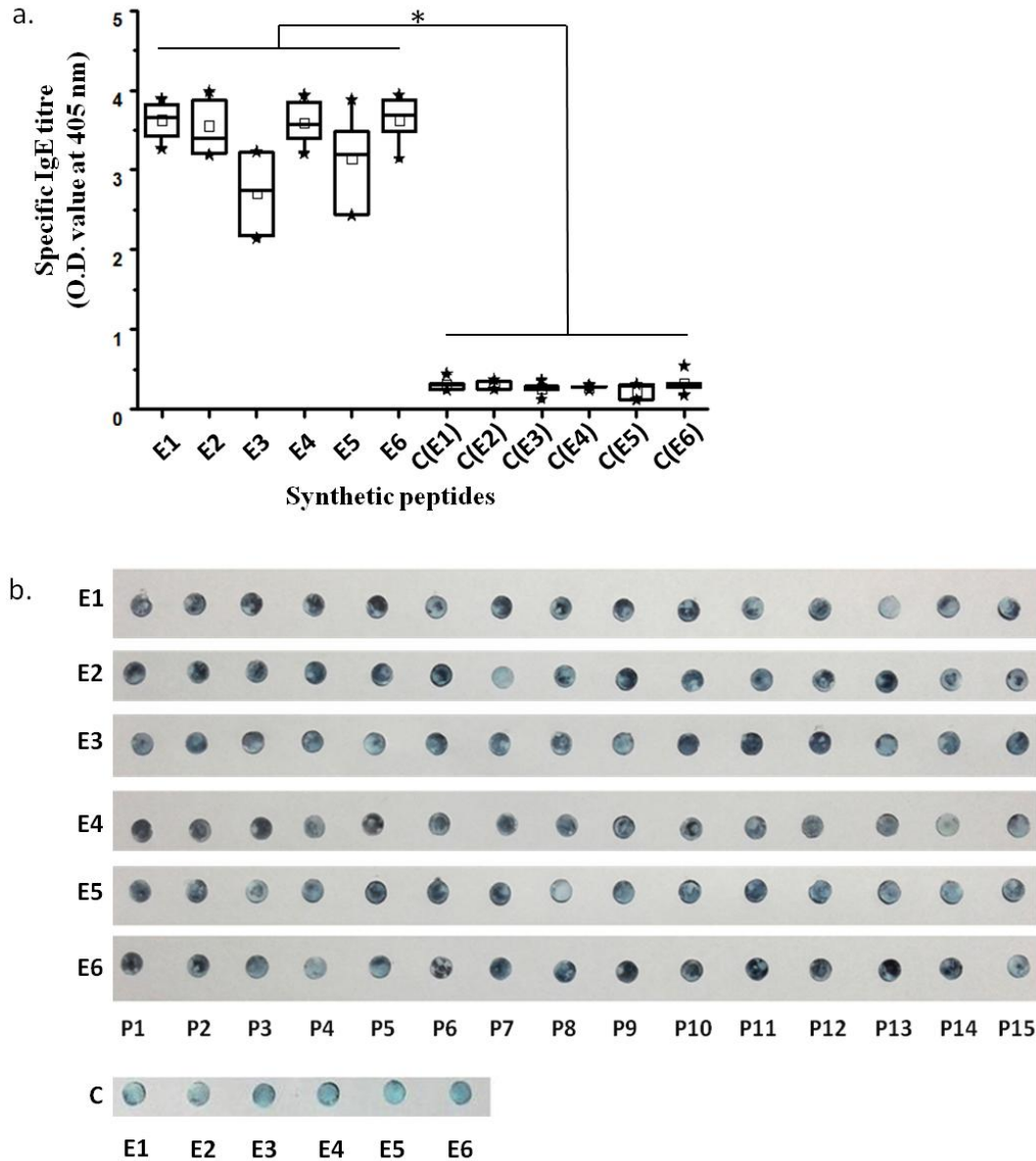


Figure S3. Immunogenicity assays with six different synthetic peptides of Asp t 36. **a.** Specific IgE titre against different synthetic epitopes using individual atopic (n=15) and healthy patients (n=3) sera. Amongst six peptides E1, E2, E4 and E6 showed maximum specific IgE titers. **b.** Dot blot immunostaining of six peptides incubated with individual atopic (n=15) and pooled healthy patients sera. C: Control. Statistical Analysis: One-way ANOVA employing Tukey's multiple comparison Test. * represents $p < 0.05$.

Aspergillus terreus	MP--RQFFVGGNGKMGNTADTSITSIKNLNDAKLDS--SSEVVISPPALYLLAREVANS	56
Triticum aestivum	MG--RKFFVGGNWKCNGTVEQVESIVNTLNAGQLASTDVVEVVVSPFYVLPTVKGLRP	58
Penaeus monodon	MSSPRKFVFIGNWKMNVDKNRINGIVKMNNNAALDA--NTEAVVGCPSCYLSYAREHLTH	58
Crangon crangon	MSGSRKFVFGGNGWKMGDKAAIDIGI VDFMKKGPLNP--NTEVVVGCPCYLSYTRKLPA	58
Scylla paramamosain	MANQRKFVFGGNGWKMGDKAAIDIGI ISFM-KGPLNA--DTEVVVGCPQCYLMYTREHMPA	57
Procambarus clarkii	MANQRKFVFGGNGWKMGDRAGDISI ISFM-KGPLSA--DTEVVVGCPQCYLMYTREHLP	57
Blattella germanica	MG--RKFFVGGNGWKMGDKGIEEIVKFLGAPLDLP--NVEVVGVPAIYLMCKNILPD	56
Dermatophagoides farinae	MG--RKFFVGGNGWKMGNKTAIKEIVDFLKNGLPDS--NVEVVGVPAIYLMCKNILPD	56
D. pteronyssinus	MG--RKFFVGGNGWKMGNSRATNDLILKTNTPLDP--NTEVVGVGPSIYLADVRLKLSK	56
Consensus	* *:*:* **:* : : : : *.: . * *: *	
Aspergillus terreus	NIGVAAQNVPDKPGAFTG----EISVEQLRDAKIDWTLGHSERRVILKESDEFIARKV	112
Triticum aestivum	EIQVAAQNCWCVKKGGAFTG----EVSAEMLVNLVGPVWLGHSERRSLMGESEFVGEKV	114
Penaeus monodon	NIGIAAQNCYKVARGNFSG----EISPEMIKDCGDWVILGHPERTVTNEPDSFIAEKV	114
Crangon crangon	EIGVAAQNCYKVAKGAFTG----EISPAMVKDCGCCEWVILGHSERRNVFNEDQLISEKV	114
Scylla paramamosain	NIGVAAQNCYKTAKGAFTG----EISPAMIKDCGCCEWVILGHSERRNVFEPQDLISEKV	113
Procambarus clarkii	NIGVAAQNCYKVAKGAFTG----EISPAMIKDCGCCEWVILGHSERRNVFNEDQLISEKV	113
Blattella germanica	NVAAAAQNCYKVPKGAFTGKHGGEISFAMLNDIGINWVILGHSERRNVFEGKDDLISDKV	116
Dermatophagoides farinae	NIRVAAQNCYKVDKGAFTG----EISPAMIKDVGAEWVILGHSERRNVGESDQLIGEKV	112
D. pteronyssinus	SIGVAAQNCYKVAKGAFTG----EISPAMIKDVGAEWVILGHSERRNVFENDQLIGEKV	112
Consensus	: : ****: * **: **: *.:** ***: * :.:**	
Aspergillus terreus	KAAIDGGVSIFVICGETLEEREAGTKTIDVVTROQLNAVAKELSKSEQWAKVVIAYEPVWAIG	172
Triticum aestivum	AYALAQGLKVLIACVGGETLEQREAGSTMVAEQQTKAIADKI--KDWNTVVVAYEPVWAIG	172
Penaeus monodon	AHAQEAGMKIIACLCTETDDRQKRTEEVLFKQLTSLAAAI--TDWSRVVLAFAELWASN	172
Crangon crangon	GHALEAGLKVIPICIGEKLEDERESNRTEEUVFAQMKAIVPNI--SDWSRVVIAEPVWAIG	172
Scylla paramamosain	GHALEAGLKVIPICIGEKLEERESNRTEEUVFAQMKAIVPNI--SDWSRVVIAEPVWAIG	171
Procambarus clarkii	GHALEAGLKVIPICIGEKLEERESNRTEEUVFAQMKAIVPNI--SDWSRVVIAEPVWAIG	171
Blattella germanica	VHALESGLNVVACIGEKLEEREGGKTEEUVFQQTKAIADKI--KDWSKVVIAYEPVWAIG	174
Dermatophagoides farinae	EHALQEGHLVHIACIGELLEEREAGKTTEEUVFRQTQVISKHV--KDWFVKVLAIEFPVWAIG	170
D. pteronyssinus	EHALNEGHLNVHIACIGELFEEREAGKTTEEUVFRQTQVISKHV--KDWSKVVIAYEPVWAIG	170
Consensus	* *: :.*: * :.:. * *: * *: * :. :*.**:***:***.	
Aspergillus terreus	TGKVATEQAQEVHAAIRKWLTETISPEVSDNTRI IYGGSVSEKNCRELAKQPVDVDFLV	232
Triticum aestivum	TGKVASPAQAQEVHANLRDWLKTNVSPEVAESTRI IYGGSVTGASKCELAAQPDVDFLV	232
Penaeus monodon	TGTFATNQPVQDALGLVRNWLRNNVSDKVADTTTLIYAGSVSSDNCREMASLENLDFLV	232
Crangon crangon	TGKTASPOQAQDVHAKLRQWLTVENVAEVAESCRI IYGGSVSPSNCAELAKMGIDGLV	232
Scylla paramamosain	TGKTATPEQAQDVHAKLRQWLDRNVSPQVAESTRI IYGGSVSAGNCKELAKTGIDGLV	231
Procambarus clarkii	TGKTATPEQAQEVHAKLRQWLDRNVVAEVAESTRI IYGGSVTPGNCKELAKTGIDGLV	231
Blattella germanica	TGKTATPEQAQDVHKKLRWLKSNVSEQVAQDTRIVYGGSVTGANCLAREADIDGLV	234
Dermatophagoides farinae	TGKTASPOQAQDVHKKLRQWLFQCFSENVSFOIAETIRI IYGGSVTANNAKELASQADVDFLV	230
D. pteronyssinus	TGKTASPOQAQEVHQRLQWFTSNISFOIAETIRI IYGGSVTASNAKELASQADVDFLV	230
Consensus	** *: *.*: .*: :.: :.: **:***: **.**:*****	
Aspergillus terreus	GGASLKPEAFVDI INARL-----	249
Triticum aestivum	GGASLKPEFIDI INAAVKS-----	253
Penaeus monodon	GGASLKPDIVDI INSRRSHVSELLTSLRIEBQQG	266
Crangon crangon	GGASLKPDFVTI INARG-----	249
Scylla paramamosain	GGASLKPDFVTI INARA-----	248
Procambarus clarkii	GGASLKPDFVQI INARD-----	248
Blattella germanica	GGASLKPEFVQI INAKN-----	251
Dermatophagoides farinae	GGASLKPEFVQI VNARQ-----	247
D. pteronyssinus	GGASLKPEFVQI VNARQ-----	247
Consensus	***** :. **: :	

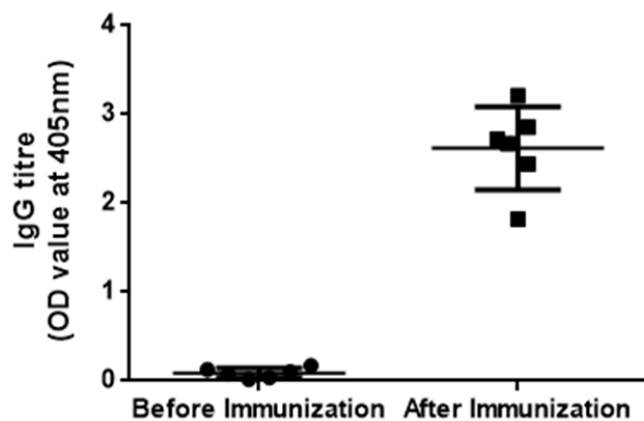


Figure S5. A comparative study on IgG level before and after immunization of rabbit with Asp t 36.

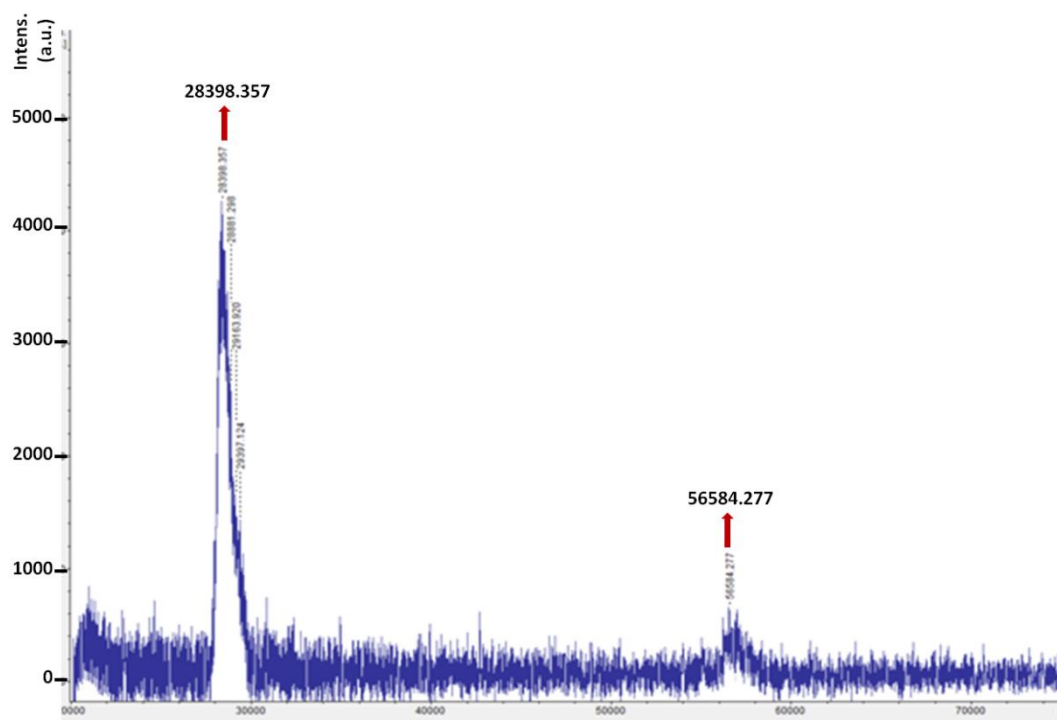


Figure S6. Mass spectra of the intact recombinant protein (rAsp t 36), purified through Ni-NTA column using MALDI TOF/TOF. Result showed a major monomeric population along with a small dimeric population of rAsp t 36.

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As this particular abbreviation is a fungal allergen name (Asp t 36) given by the WHO/IUIS, it should not be included in the abbreviation list of the manuscript.